



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

### Specific binding of an antigen-antibody complex to apoptotic human neutrophils

**Citation for published version:**

Hart, SP, Jackson, C, Kremmel, LM, McNeill, MS, Jersmann, H, Alexander, KM, Ross, JA & Dransfield, I  
2003, 'Specific binding of an antigen-antibody complex to apoptotic human neutrophils', *The American Journal of Pathology*, vol. 162, no. 3, pp. 1011-8. [https://doi.org/10.1016/S0002-9440\(10\)63895-3](https://doi.org/10.1016/S0002-9440(10)63895-3)

**Digital Object Identifier (DOI):**

[10.1016/S0002-9440\(10\)63895-3](https://doi.org/10.1016/S0002-9440(10)63895-3)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Publisher's PDF, also known as Version of record

**Published In:**

The American Journal of Pathology

**General rights**

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



# Specific Binding of an Antigen-Antibody Complex to Apoptotic Human Neutrophils

Simon P. Hart,\* Caroline Jackson,\*  
L. Maximilian Kremmel,\* Mary S. McNeill,\*  
Hubertus Jersmann,\* Karen M. Alexander,\*  
James A. Ross,<sup>†</sup> and Ian Dransfield\*

From the Medical Research Council Centre for Inflammation Research,\* University of Edinburgh Medical School, Edinburgh; and the Edinburgh University Department of Surgery,<sup>†</sup> Royal Infirmary, Edinburgh, Scotland

**Examination of apoptotic cell surface molecules has so far failed to reveal cell type-specific membrane alterations that serve as a signal for phagocytosis. In the present study we have identified a novel murine monoclonal antibody, BOB93, which bound to the surface of apoptotic neutrophils but not to apoptotic lymphocytes. BOB93 binding to apoptotic neutrophils was dependent on the presence of the sialoglycoprotein fetuin, a constituent of bovine serum. We demonstrate that fetuin is the antigen for BOB93, and that BOB93 and fetuin form a complex in solution that is necessary and sufficient for binding to apoptotic neutrophils. Individuals who were homozygous for an adenine nucleotide at position 519 of the gene for the immune complex receptor FcγRIIA exhibited markedly reduced binding of BOB93/fetuin. This report is the first to provide evidence that antigen-antibody complexes bind specifically to apoptotic neutrophils and implicates apoptosis-associated changes in Fcγ receptor function. (*Am J Pathol* 2003, 162:1011–1018)**

Neutrophils have been implicated in the pathogenesis of a variety of inflammatory diseases including the adult respiratory distress syndrome, idiopathic pulmonary fibrosis, ulcerative colitis, and rheumatoid arthritis.<sup>1</sup> Although the neutrophil is a vital component of the body's defense against infectious agents, uncontrolled release of its formidable array of toxic substances may inflict "friendly fire" damage on surrounding tissue and propagate the inflammatory response, leading to scarring and tissue destruction.<sup>2</sup> The fate of recruited neutrophils, which are present in large numbers at a site of inflammation, is apoptosis<sup>3</sup> culminating in recognition and safe disposal of the dying cells by phagocytes.<sup>4,5</sup> Neutrophil apoptosis is associated with down-regulation of potentially harmful cellular functions, such as stimulated release of granule contents,<sup>6,7</sup> and leads to surface mem-

brane alterations that signal noninflammatory phagocytic clearance by macrophages.

Efficient removal of apoptotic cells before release of their potentially harmful intracellular contents is critical because if excessive apoptotic cell load occurs, development of autoimmune or chronic inflammatory pathology may ensue.<sup>8,9</sup> The sheer diversity of surface molecules that have been proposed to be involved in phagocyte recognition of apoptotic cells implies that phagocyte recognition signals are complex and unlikely to depend on a single molecule.<sup>5</sup> The molecular alterations on the surface of apoptotic cells that are responsible for phagocyte recognition also remain to be fully characterized. Many studies have implicated exposure of the anionic phospholipid phosphatidylserine on the apoptotic cell membrane as an important determinant of phagocyte recognition.<sup>10–12</sup> We and others have previously shown that a number of alterations in the protein and carbohydrate composition of the plasma membrane are associated with apoptosis.<sup>7,13–16</sup> It has also become apparent that apoptosis is associated with membrane alterations that confer specific binding of plasma proteins, with the potential for opsonization and regulation of subsequent phagocyte recognition. In particular, there is evidence that the collectin family of molecules, including complement component C1q,<sup>17</sup> mannose binding lectin,<sup>18</sup> and surfactant protein A,<sup>19</sup> exhibit specific binding to apoptotic cells. However, the binding of complement components may be a relatively late event in the apoptotic process or may even reflect the presence of necrotic cells.<sup>20</sup> Recently, IgM was shown to bind to cell surface lysophospholipids on apoptotic Jurkat cells via its Fab' portions, providing a mechanism for complement binding to apoptotic cells.<sup>21</sup> Other proteins that may bind to apoptotic cells include the acute phase proteins pentraxin-3,<sup>22</sup> serum amyloid P,<sup>23</sup> and C-reactive protein.<sup>24</sup> However, our data demonstrating augmentation of phagocytosis of apoptotic neutrophils, but not lymphocytes, after ligation of macrophage CD44 indicates that surface determinants of subsequent phagocytic clearance may be specific to certain cell lineages. We have therefore undertaken further studies to characterize changes in the surface expression of carbohydrates and proteins associated with neutrophil

Supported by The Medical Research Council and a University of Edinburgh Faculty of Medicine Fellowship.

Accepted for publication December 3, 2002.

Address reprint requests to Dr S. P. Hart, MRC Centre for Inflammation Research, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, UK. E-mail: s.hart@ed.ac.uk.

apoptosis using dual-color flow cytometric analysis.<sup>15,16</sup> We now report the binding characteristics of a unique monoclonal antibody, termed BOB93, which displayed specific binding to apoptotic neutrophils.

## Materials and Methods

### Antibodies and Other Reagents

Cell culture materials and fetal calf serum (FCS) were from Invitrogen (Paisley, UK) and Percoll was from Pharmacia (Little Chalfont, UK). Monoclonal antibody (mAb) BOB93 (IgG<sub>1</sub> isotype) was prepared by fusion of splenocytes from a BALB/c mouse immunized with the human myelomonocytic cell line THP-1 (obtained from the ECACC, Porton Down, UK) with Sp2/O'-Ag14 (ECACC) nonsecreting myeloma cell line. Fusion products secreting immunoglobulin (Ig) were tested in flow cytometry for reactivity with apoptotic neutrophils and subcloned twice before further analysis. 3G8 mAb (anti-CD16) was the gift of Dr. J. Unkeless, Mount Sinai Medical School, New York, NY. Fluorescein isothiocyanate (FITC)-conjugated 3G8 was prepared as described previously<sup>15</sup> and used at a final concentration of 2  $\mu$ g/ml. Control mAb hybridoma MOPC21C (IgG<sub>1</sub>) was obtained from the ECACC and grown in Dulbecco's modified Eagle's medium plus 10% FCS. Fetuin, asialofetuin, and bovine serum albumin were from Sigma (Poole, UK).  $\alpha_2$ -HS glycoprotein and  $\alpha_1$ -acid glycoprotein were from Calbiochem (CN Biosciences, Nottingham, UK). Complexes of murine IgG<sub>1</sub> were formed by combining 500  $\mu$ g/ml of biotinylated albumin (Sigma) with 85  $\mu$ g/ml of FITC-conjugated anti-biotin IgG<sub>1</sub> (clone BN-34, Sigma) in phosphate-buffered saline (PBS) for 30 minutes on ice, and then diluted in PBS before use in neutrophil-binding assays.

### Protein Labeling

Fetuin was dissolved at 2.5 mg/ml in PBS and dialyzed against 100 mmol/L of sodium bicarbonate, pH 8.2. FITC (Sigma) was dissolved at 1.5 mg/ml in dimethyl sulfoxide and added dropwise to a total volume of 45  $\mu$ l per ml of protein solution. The mixture was then incubated for 2 hours at room temperature in the dark. The FITC-protein solution was finally gel filtered on a PD-10 column (Pharmacia) that had been equilibrated with PBS.

### Cell Isolation

Leukocytes were isolated from human peripheral blood by dextran sedimentation and discontinuous Percoll gradient centrifugation as described.<sup>25</sup> Neutrophils were cultured at  $4 \times 10^6$ /ml in Iscove's modification of Dulbecco's modified Eagle's medium containing 10% autologous serum at 37°C in a 95% air/5% CO<sub>2</sub> atmosphere for 20 hours, during which time a proportion of the cells underwent apoptosis.<sup>3</sup> Lymphocytes were isolated by adherence and negative selection from the mononuclear cell band, and apoptosis was induced by 20-hour culture in RPMI 1640 in the absence of serum.

### Flow Cytometry

Indirect immunofluorescence was used to assess antibody binding to leukocytes using FITC-conjugated F(ab')<sub>2</sub> goat anti-mouse Ig (DAKO, Ely, UK), and flow cytometric analysis was performed as previously described.<sup>7,15</sup> FITC-fetuin was incubated with cells in PBS for 30 minutes before washing. Counterstaining with annexin V-PE (Caltag, Towchester, UK) was used to identify apoptotic cells within the aged neutrophil population. All neutrophil populations were routinely tested for exclusion of propidium iodide and were >99% propidium iodide-negative.

### Dot Blotting

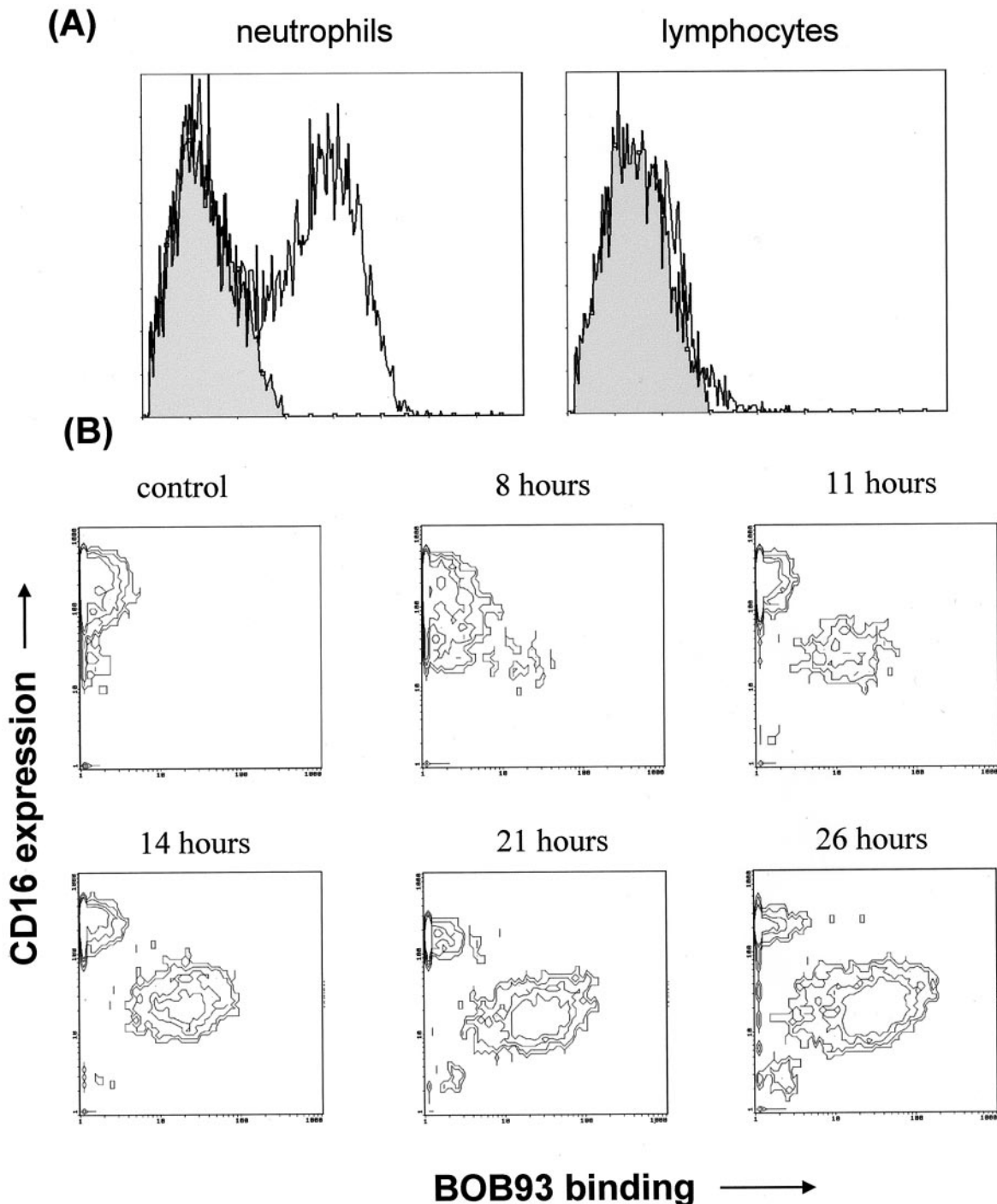
Two hundred- $\mu$ l samples containing proteins at 1.25  $\mu$ g/ml or 1:80 dilutions of serum in PBS were applied to a nitrocellulose membrane using a dot-blot manifold. The membrane was blocked with PBS/0.1% Tween 20 and then incubated with 1:100 BOB93 serum-free supernatant for 30 minutes at room temperature. After washing the membrane was incubated with 1:4000 goat anti-mouse Ig-horseradish peroxidase (DAKO) and developed with enhanced chemiluminescence (Amersham, Little Chalfont, UK).

### Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

BOB93 in serum-free supernatant was ultracentrifuged at  $300,000 \times g$  in a Beckman Optima TLX ultracentrifuge to remove immunoglobulin aggregates. The supernatant was then mixed with fetuin or bovine serum albumin (control) at a final concentration of 0.5 mg/ml for 30 minutes on ice, before ultracentrifugation at  $300,000 \times g$  for 30 minutes. The supernatant was collected and the pellet was washed once in PBS and resolubilized. Aliquots of supernatants and pellets were run on a denaturing 9% polyacrylamide gel, electroblotted onto nitrocellulose, probed with 1:4000 horseradish peroxidase-labeled anti-mouse Ig (DAKO), and visualized by enhanced chemiluminescence (Amersham).

### Fc $\gamma$ RIIA Genotyping

Genomic DNA was extracted from freshly isolated neutrophils by lysis in 0.1% SDS, 10 mmol/L Tris-HCl, pH 8, and 1 mmol/L ethylenediaminetetraacetic acid, followed by incubation with 100  $\mu$ g/ml of RNase (Sigma) for 60 minutes at 37°C. Proteins were precipitated with potassium acetate and DNA was precipitated from the supernatant in isopropanol and then ethanol. A 449-bp fragment containing the polymorphism at position 519 in exon 4 of the Fc $\gamma$ RIIA gene was amplified by polymerase chain reaction using a 50- $\mu$ l reaction mixture containing 20 pmol of forward primer 5'-TGA GAC TGA AAA ACC CTT GG-3'; 20 pmol of reverse primer 5'-CAC TCC TCT TTG



**Figure 1.** Monoclonal antibody BOB93 binds to apoptotic neutrophils. **A:** Apoptosis was induced in human peripheral blood neutrophils and lymphocytes during *in vitro* culture. Cells were labeled with BOB93 hybridoma supernatant followed by F(ab')<sub>2</sub> FITC-labeled goat anti-mouse Ig antibody, and analyzed by flow cytometry (**shaded histogram**, control IgG<sub>1</sub> antibody; **open histogram**, BOB93). Approximately 50% of cells in each population were apoptotic as assessed by annexin V binding. **B:** BOB93 in hybridoma supernatant bound selectively to CD16<sup>low</sup> apoptotic cells within a population of aged neutrophils. Dual-immunofluorescence analysis was performed using BOB93, PE-conjugated F(ab')<sub>2</sub> anti-mouse Ig, and FITC-conjugated CD16 mAb 3G8. FL1 (y axis) represents CD16 expression and FL2 (x axis) represents BOB93 binding. There was a time-dependent increase in neutrophil apoptosis during *in vitro* culture and BOB93 bound selectively to the apoptotic cells. Data are from a single experiment representative of three that were performed.

CTC CAG TG-3'; 1.5 mmol/L of MgCl<sub>2</sub>; 2.5 U of *Taq*DNA polymerase (Promega) in buffer A (Promega); 200 μmol/L each of dNTPs (Promega, Southampton, UK); and 1 μl of DNA. The following cycling conditions were used: 35 cycles each comprising 30-second segments of 94°C for

30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, followed by a 5-minute final extension at 72°C. Ten-μl of each product was run on a 1% agarose gel containing ethidium bromide and photographed under UV illumination to confirm successful amplification. Each



product was then purified using a Qiagen polymerase chain reaction purification kit and sequenced by MWG-Biotech (Milton Keynes, UK).

## Results

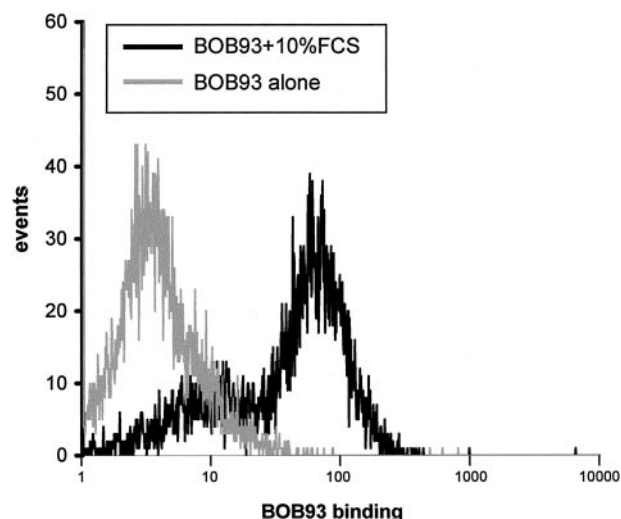
### *Antibody BOB93 Binds Specifically to Apoptotic Neutrophils*

A large number of antibodies were screened for their binding properties to apoptotic neutrophils using dual-color indirect immunofluorescence and flow cytometric analysis.<sup>7,15,16</sup> We identified one mAb cell culture supernatant, BOB93, that bound specifically to a subpopulation of neutrophils that had been cultured *in vitro* (Figure 1A), but exhibited very low levels of binding to freshly isolated neutrophils. BOB93-positive neutrophils bound annexin V, confirming that BOB93-labeled cells represented apoptotic neutrophils. To determine whether BOB93 binding reflected a neutrophil-specific membrane alteration associated with apoptosis we also examined human peripheral blood lymphocytes that had been induced to undergo apoptosis during serum-free culture. In contrast with neutrophils, apoptotic lymphocytes exhibited only a single BOB93<sup>low</sup> population (Figure 1A). Temporal analysis revealed that binding of BOB93 to neutrophils closely mirrored CD16 shedding (Figure 1B), indicating a tight association between expression of the BOB93 binding site and other surface molecular alterations associated with apoptosis. Thus, binding of BOB93 defines a neutrophil-specific surface molecular alteration that accompanies apoptosis.

### *BOB93 Binding Is Dependent on a Component of Bovine Serum*

The specific binding of BOB93 to apoptotic neutrophils led us to further characterize the nature of the antigen recognized by the BOB93 mAb. Initial screening was performed using antibodies in hybridoma supernatant that contains growth medium and 10% FCS. However, in experiments using serum-free BOB93 hybridoma supernatant we did not observe binding of BOB93 to apoptotic neutrophils, despite the presence of similar levels of antibody. Reconstitution of serum-free BOB93 supernatant with 10% FCS fully restored binding to apoptotic neutrophils (Figure 2).

Because BOB93 failed to bind apoptotic neutrophils in the absence of serum but FCS was able to restore cellular reactivity, we proposed that a component of FCS was required for antibody binding to apoptotic neutrophils. We therefore tested whether BOB93 was able to bind to proteins present in FCS. Dot-blot analysis confirmed that BOB93 bound to FCS and to fetuin, one of its principal protein constituents (Figure 3). Although fetuin displays significant sequence homology between the many different species in which it has been identified,<sup>26</sup> BOB93 did not bind to rabbit or human serum or to the human fetuin homolog  $\alpha_2$ -HS glycoprotein. Furthermore, we did not



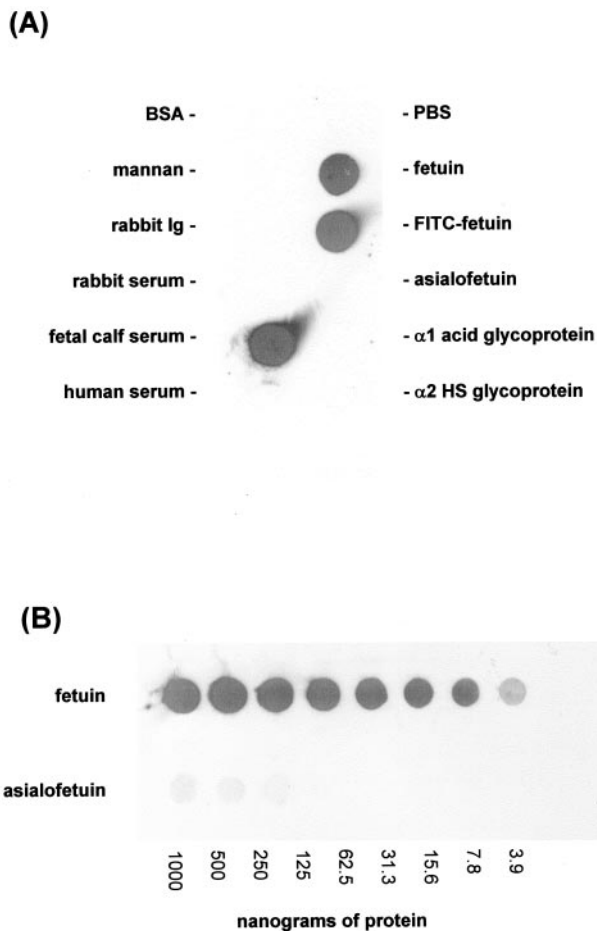
**Figure 2.** Bob93 binding requires a constituent of bovine serum. BOB93 in serum-free medium was incubated with aged neutrophils in the presence or absence of 10% FCS. The cells were washed and BOB93 binding was detected by anti-mouse Ig-FITC. In the absence of serum BOB93 did not bind aged neutrophils. Binding was reconstituted by addition of 10% fetal calf serum. A representative flow cytometry histogram is shown.

observe binding of BOB93 to asialofetuin, suggesting that sialic acid-containing oligosaccharides present on bovine fetuin are an important determinant of antibody binding. However, sialylation does not universally confer this property because BOB93 failed to bind the highly sialylated  $\alpha_1$ -acid glycoprotein or to sialylated bovine serum albumin (Figure 3 and data not shown). We have been unable to further identify the epitope recognized by BOB93 because unfolding of fetuin in SDS or digestion of fetuin with trypsin both resulted in loss of antigenicity and BOB93 did not bind to oligosaccharides prepared from fetuin with sufficient affinity to allow further characterization of potential epitopes (data not shown). Together our data suggested that BOB93 recognizes fetuin in a sialic acid-dependent and species-specific manner.

### *Fetuin Is Required for BOB93 Binding*

Like FCS (Figure 2), fetuin restored binding of serum-free BOB93 to apoptotic neutrophils (Figure 4A), whereas control proteins albumin or asialofetuin had no effect (data not shown). Peak binding of BOB93 was observed in the presence of  $\sim 0.3$  mg/ml of fetuin, which is similar to its concentration in 10% FCS and thus in hybridoma culture supernatant.<sup>27</sup>

A possible explanation for these findings was that fetuin was able to bind to the apoptotic cell surface, which consequently conferred BOB93 binding. We performed preincubation and co-incubation experiments to directly test this hypothesis. Aged neutrophils that had been incubated with 0.3 mg/ml of fetuin and then washed did not exhibit BOB93 binding, whereas co-incubation of 0.3 mg/ml of fetuin with BOB93 permitted binding of the antibody (Figure 4B, top). This result suggested that fetuin alone could not bind to apoptotic neutrophils. For confirmation we performed binding studies with fetuin



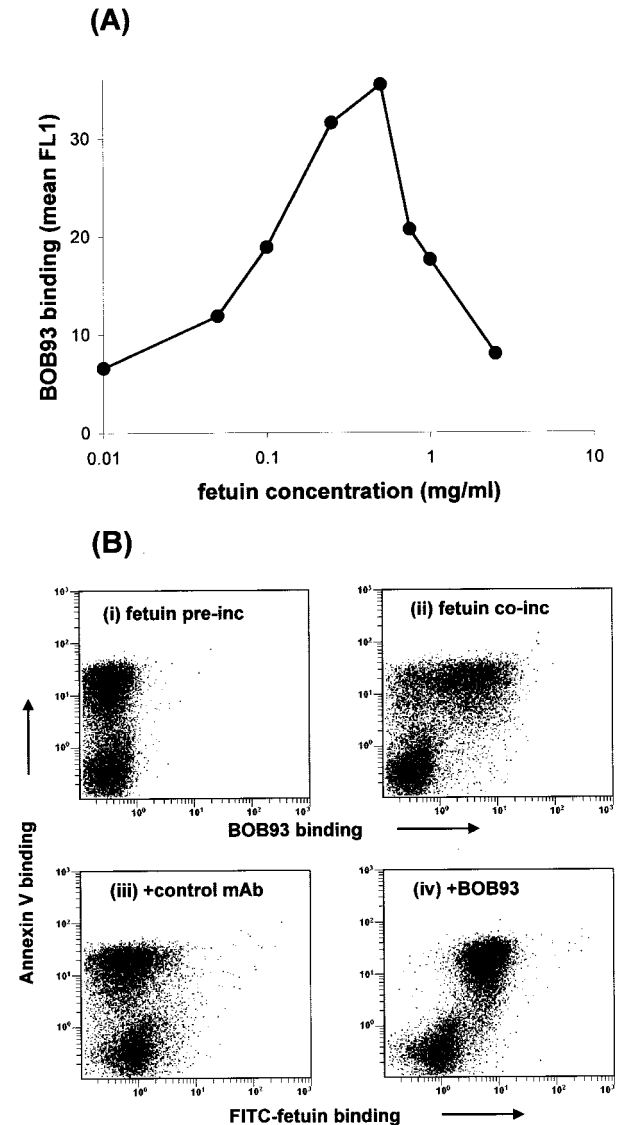
**Figure 3.** Fetuin is the BOB93 antigen. **A:** Proteins (1  $\mu$ g total protein) were applied to nitrocellulose and allowed to dry. The membrane was blocked with PBS/0.1% Tween 20 and then probed with BOB93 followed by goat anti-mouse Ig-horseradish peroxidase. The membrane was developed using enhanced chemiluminescence (Amersham). BOB93 bound specifically to FCS and fetuin. **B:** Specific binding of BOB93 to fetuin was demonstrated using serial dilutions of fetuin and asialofetuin applied to nitrocellulose, probed, and developed as above.

that had been conjugated with FITC. FITC-labeled fetuin did not bind to aged neutrophils, but co-incubation with unlabeled BOB93 resulted in FITC-fetuin binding to apoptotic neutrophils (Figure 4B, bottom). These results demonstrated conclusively that fetuin alone is unable to bind to neutrophils, but that a combination of fetuin and BOB93 was required to bind to the apoptotic neutrophil surface.

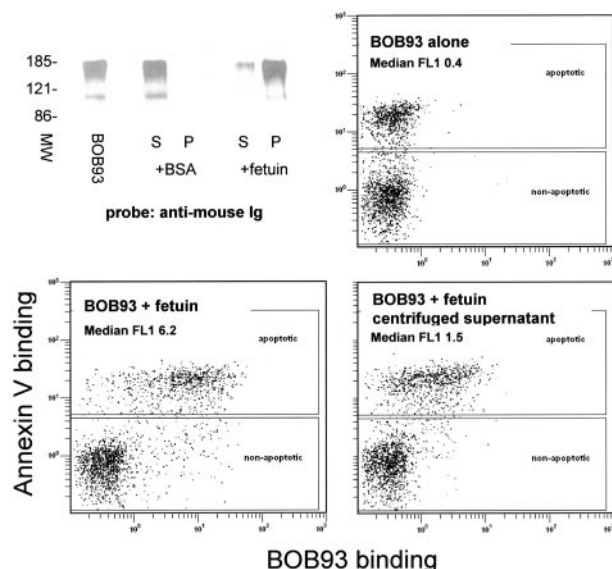
### *BOB93 and Fetuin Form a Complex in Solution*

Because both BOB93 antibody and fetuin were necessary for either to bind to apoptotic neutrophils, we reasoned that an antigen-antibody complex was formed in solution that permitted cell binding. To test this hypothesis, BOB93 and fetuin were mixed and then ultracentrifuged at  $300,000 \times g$  to pellet any complexes that had been formed. The presence of BOB93 antibody in the resulting pellet or supernatant was detected by SDS-PAGE and Western blotting using a peroxidase-labeled anti-mouse Ig probe. Figure 5 demonstrates that after

incubation with fetuin the majority of BOB93 is present in the ultracentrifuged pellet with very little remaining in the supernatant, implying that a complex is indeed formed between BOB93 and fetuin. We tested the supernatants before and after ultracentrifugation for their ability to bind to apoptotic neutrophils. Whereas the BOB93/fetuin starting material was able to bind apoptotic neutrophils, the postcentrifugation supernatant exhibited markedly reduced cell binding (Figure 5). Our interpretation of this



**Figure 4.** Binding of serum-free BOB93 is reconstituted by fetuin. **A:** Serum-free BOB93 was incubated with aged human neutrophils in the presence of increasing concentrations of fetuin and binding was assessed by flow cytometry. The mean fluorescence of the BOB93<sup>high</sup> apoptotic population was recorded. In this representative experiment the mean relative fluorescence of cells in the presence of control antibody was 4.0. **B:** Aged human neutrophils were preincubated with fetuin (i) or PBS (ii), washed twice, and then incubated with BOB93 alone (i) or BOB93 + fetuin 0.3 mg/ml (ii). Detection was with anti-mouse Ig-FITC. BOB93 did not bind to cells that were preincubated with fetuin, but co-incubation with fetuin permitted binding to apoptotic neutrophils. **Bottom:** FITC-labeled fetuin (0.3 mg/ml) was incubated with aged neutrophils in the presence of control antibody (iii) or BOB93 (iv). FITC-fetuin bound to apoptotic neutrophils only in the presence of BOB93. Dot plots are shown from a representative experiment using annexin V-PE to label apoptotic neutrophils.



**Figure 5.** Fetuin and BOB93 form a complex in solution. Serum-free BOB93 hybridoma supernatant was ultracentrifuged at  $300,000 \times g$  to remove immunoglobulin aggregates. The supernatant was then mixed with fetuin or bovine serum albumin (control) at a final concentration of 0.3 mg/ml for 30 minutes on ice before ultracentrifugation for 30 minutes. The supernatant was collected and the pellet was washed once in PBS and resuspended. Aliquots of supernatants (S) and pellets (P) were analyzed by SDS-PAGE and Western blotting with horseradish peroxidase-labeled anti-mouse Ig, and visualized by enhanced chemiluminescence. After incubation with fetuin, the majority of BOB93 antibody is detected in the pellet, with little remaining in the supernatant. Aliquots of BOB93 alone and BOB93 plus fetuin before and after ultracentrifugation were then tested for binding to apoptotic neutrophils using anti-mouse Ig-FITC as a second layer. The supernatant after ultracentrifugation exhibited significantly diminished BOB93 binding to apoptotic neutrophils compared with the starting material.

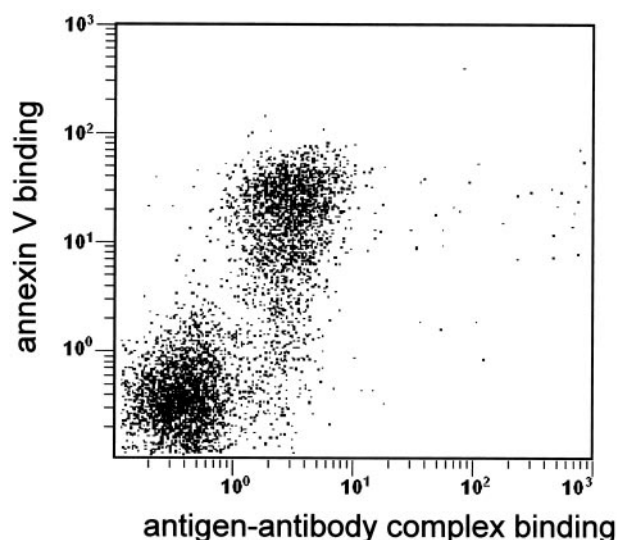
result is that a complex of BOB93 and fetuin is necessary and sufficient to bind to apoptotic neutrophils.

### *Biotinylated Albumin-Anti-Biotin Complexes Bind to Apoptotic Neutrophils*

To demonstrate that binding of an antigen-antibody complex to apoptotic neutrophils was not specific for BOB93/fetuin, we used biotinylated albumin as a multivalent antigen and mixed it with FITC-conjugated anti-biotin murine IgG<sub>1</sub> to generate labeled antigen-antibody complexes *in vitro*. When aged human neutrophils were incubated with these complexes, we observed a pattern of preferential binding to apoptotic neutrophils that was very similar to that seen with BOB93/fetuin (Figure 6).

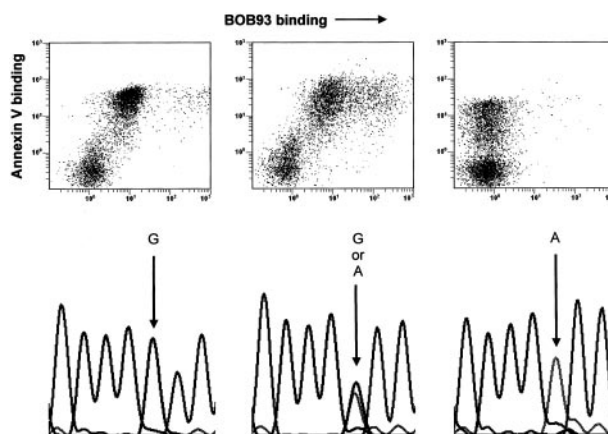
### *BOB93/Fetuin Binding Is Determined by a Common Polymorphism in FcγRIIA*

During the course of these studies we obtained reproducible results using cells from more than 50 healthy volunteer blood donors. However, we identified a small number of individuals whose apoptotic neutrophils repeatedly failed to bind BOB93/fetuin. Because our data suggested that a complex of BOB93 antibody and its antigen, fetuin, was required for cell binding, we wondered whether these negative donors were equivalent to



**Figure 6.** Apoptotic neutrophils bind complexes of biotinylated albumin and anti-biotin IgG<sub>1</sub>. Aged human neutrophils were incubated with complexes of biotinylated bovine albumin (20 μg/ml) and FITC-labeled anti-biotin murine IgG<sub>1</sub>, co-labeled with annexin V-PE, and analyzed by flow cytometry.

the low-responder individuals previously identified in studies of binding of complexed murine IgG<sub>1</sub> to viable human leukocytes.<sup>28</sup> This phenomenon has been shown to be because of a common polymorphism in the immune complex receptor FcγRIIA.<sup>29,30</sup> We extracted genomic DNA from neutrophils from a subset of our blood donors and amplified the DNA sequence containing the FcγRIIA polymorphism using polymerase chain reaction. The polymerase chain reaction product was then sequenced, and the sequence compared with BOB93/fetuin-binding data. These experiments confirmed that our negative donors did indeed exhibit the low-responder genotype, having an adenine (A) nucleotide at position 519 of the FcγRIIA gene, which leads to substitution of an arginine residue by a histidine in the second Ig-like domain of the receptor. All of our other donors had a guanine (G) or G/A (Figure 7), and apoptotic neutrophils from G/G homozy-



**Figure 7.** BOB93-negative donors have the low-responder polymorphism in FcγRIIA (CD32). Dot plots showing BOB93/fetuin binding to aged neutrophils from three different donors (top) are presented along with corresponding sequencing chromatograms showing a common polymorphism in FcγRIIA (bottom). BOB93-negative donors (right) were homozygous for an adenine (A) nucleotide at position 519 of the FcγRIIA gene.



gotes and G/A heterozygotes bound BOB93/fetuin to a similar extent.

## Discussion

With the exception of phosphatidylserine exposure, significant positive surface changes on the apoptotic cell surface that might determine recognition by phagocytes have only recently been reported. Binding of blood constituents such as complement components and acute-phase proteins to apoptotic cells may allow opsonization that will confer recognition by phagocyte receptors (eg, C1qR, CR3, and so forth). *In vivo* it is likely that the inflammatory milieu will be populated with numerous substances that could potentially opsonize apoptotic cells, in contrast to many *in vitro* models of phagocytosis of apoptotic cells that have used a basic serum-free environment (to allow the roles of different receptors to be examined). In the present study we have identified a novel monoclonal antibody, BOB93, which in the hybridoma supernatant binds specifically to apoptotic neutrophils, but not to the apoptotic lymphocytes. The antigen recognized by BOB93 was identified as fetuin, a sialylated bovine glycoprotein that is abundantly present in fetal serum.<sup>31</sup> Further characterization revealed that the BOB93 antibody alone failed to bind the apoptotic neutrophil surface, but that binding was dependent on the formation of a complex between BOB93 and fetuin.

An important clue to the molecular mechanism of BOB93/fetuin binding to apoptotic neutrophils arose from our observation of occasional negative donors whose cells reproducibly failed to bind BOB93/fetuin. Because a complex between a murine antibody and its antigen was responsible for cell binding, we hypothesized that our negative donors may be equivalent to the low-responder individuals in studies of complexed murine IgG<sub>1</sub> binding to leukocytes. Genotyping the Fc $\gamma$ RIIA polymorphism of a number of our volunteers confirmed that the negative donors possessed the low-responder genotype, being homozygous for an A nucleotide at position 519 of the gene. All of the positive donors that we genotyped were either G/G homozygotes or G/A heterozygotes. The genotyping result is important however because it implicates neutrophil Fc $\gamma$  receptors in the binding of antigen-antibody complexes to apoptotic neutrophils. The peripheral blood lymphocytes used in the present study did not exhibit significant expression of any Fc receptors (data not shown). Neutrophils, however, express two receptors for complexed IgG, Fc $\gamma$ RIIA (CD32) and Fc $\gamma$ RIIIB (CD16), but do not express significant amounts of the high-affinity receptor Fc $\gamma$ RI (CD64) under resting conditions.<sup>32</sup> It is well documented that during apoptosis neutrophils lose the majority of their Fc $\gamma$ RIIIB molecules,<sup>15</sup> and Fc $\gamma$ RIIA expression is also significantly reduced on apoptotic neutrophils.<sup>16</sup> We were therefore surprised that our data pointed to a role for Fc $\gamma$  receptors in the binding of BOB93/fetuin complexes to apoptotic neutrophils. In particular, we observed little binding of BOB93/fetuin to nonapoptotic neutrophils, implying that there may be apoptosis-associated changes in Fc $\gamma$  receptor function.

Clearly this observation requires further investigation, but the novel findings reported here have several important implications for the removal of apoptotic neutrophils from inflammatory sites, and thus for disease pathogenesis. Although the process of phagocyte clearance of apoptotic cells has been extensively studied under serum-free conditions *in vitro*, the presence of serum components including IgG may significantly influence the way in which phagocytes recognize apoptotic cells. It has been shown that apoptotic cell clearance fails to stimulate release of proinflammatory mediators by macrophages *in vitro*.<sup>33,34</sup> In many inflammatory diseases antigen-antibody complexes may be found in the bloodstream or in the tissues (eg, in the joint in rheumatoid arthritis) where they contribute to the inflammatory process by activating Fc receptor-bearing leukocytes. Our data raise the intriguing possibility that antigen-antibody complexes may specifically opsonize apoptotic neutrophils and consequently modulate the mechanism by which they are recognized and phagocytosed by macrophages.

## Acknowledgment

We thank Dr. Tony Merry, University of Oxford, UK, for supplying fetuin-derived oligosaccharides for use in binding competition assays.

## References

- Weiss SJ: Mechanisms of disease: tissue destruction by neutrophils. *N Engl J Med* 1989, 320:365–376
- Haslett C: Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 1999, 160: S5–S11
- Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C: Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 1989, 83:865–875
- Savill JS, Fadok VA, Henson PM, Haslett C: Phagocyte recognition of cells undergoing apoptosis. *Immunol Today* 1993, 14:131–136
- Giles KM, Hart SP, Haslett C, Rossi AG, Dransfield I: An appetite for apoptotic cells: controversies and challenges. *Br J Haematol* 2000, 109:1–12
- Whyte MKB, Meagher LC, MacDermot J, Haslett C: Impairment of function in aging neutrophils is associated with apoptosis. *J Immunol* 1993, 150:5124–5134
- Dransfield I, Stocks SC, Haslett C: Regulation of cell adhesion molecule expression and function associated with neutrophil apoptosis. *Blood* 1995, 85:3264–3273
- Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S: Lethal effect of the anti-Fas antibody in mice. *Nature* 1993, 364:806–809
- Hagimoto N, Kuwano K, Miyazaki H, Kunitake R, Fujita M, Kawasaki M, Kaneko Y, Hara N: Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. *Am J Respir Cell Mol Biol* 1997, 17:272–278
- Verhove B, Schlegel RA, Williamson P: Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J Exp Med* 1995, 182:1597–1601
- Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM: Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 1992, 148:2207–2216
- Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM: A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 2000, 405:85–90



13. Duvall E, Wyllie AH, Morris RG: Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology* 1985, 56:351–358
14. Jones J, Morgan BP: Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: functional relevance and role in inflammation. *Immunology* 1995, 86:651–660
15. Dransfield I, Buckle A-M, Savill JS, McDowall A, Haslett C, Hogg N: Neutrophil apoptosis is associated with a reduction in CD16 (Fc $\gamma$ RIII) expression. *J Immunol* 1994, 153:1254–1263
16. Hart SP, Ross JA, Ross K, Haslett C, Dransfield I: Molecular characterization of the surface of apoptotic neutrophils: implications for functional downregulation and recognition by phagocytes. *Cell Death Differ* 2000, 7:493–503
17. Korb LC, Ahearn JM: C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *J Immunol* 1997, 158:4525–4528
18. Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM: C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* 2001, 194:781–795
19. Schagat TL, Wofford JA, Wright JR: Surfactant protein A enhances alveolar macrophage phagocytosis of apoptotic neutrophils. *J Immunol* 2001, 166:2727–2733
20. Gaipal US, Kuenkele S, Voll RE, Beyer TD, Kolowos W, Heyder P, Kalden JR, Herrmann M: Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death. *Cell Death Differ* 2001, 8:327–334
21. Kim SJ, Gershov D, Ma X, Brot N, Elkon KB: I-PLA(2) Activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. *J Exp Med* 2002, 196:655–665
22. Rovere P, Peri G, Fazzini F, Bottazzi B, Doni A, Bondanza A, Zimmermann VS, Garlanda C, Fascio U, Sabbadini MG, Rugarli C, Mantovani A, Manfredi AA: The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells. *Blood* 2000, 96:4300–4306
23. Familian A, Zwart B, Huisman HG, Rensink I, Roem D, Hordijk PL, Aarden LA, Hack CE: Chromatin-independent binding of serum amyloid P component to apoptotic cells. *J Immunol* 2001, 167:647–654
24. Gershov D, Kim S, Brot N, Elkon KB: C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med* 2000, 192:1353–1364
25. Ackerman SK, Douglas SD: Purification of human monocytes on microexudate-coated surfaces. *J Immunol* 1978, 120:1372–1374
26. Brown WM, Saunders NR, Mollgard K, Dziegielewska KM: Fetuin—an old friend revisited. *Bioessays* 1992, 14:749–755
27. Nie Z: Fetuin: its enigmatic property of growth promotion. *Am J Physiol* 1992, 263:C551–C562
28. Leeuwenberg JF, van de Winkel JG, Jeunhomme TM, Buurman WA: Functional polymorphism of IgG FcRII (CD32) on human neutrophils. *Immunology* 1990, 71:301–304
29. Warmerdam PA, van de Winkel JG, Gosselin EJ, Capel PJ: Molecular basis for a polymorphism of human Fc gamma receptor II (CD32). *J Exp Med* 1990, 172:19–25
30. Clark MR, Stuart SG, Kimberly RP, Ory PA, Goldstein IM: A single amino acid distinguishes the high-responder from the low-responder form of Fc receptor II on human monocytes. *Eur J Immunol* 1991, 21:1911–1916
31. Dziegielewska KM, Matthews N, Saunders NR, Wilkinson G: Alpha 2HS-glycoprotein is expressed at high concentration in human fetal plasma and cerebrospinal fluid. *Fetal Diagn Ther* 1993, 8:22–27
32. Buckle AM, Jayaram Y, Hogg N: Colony-stimulating factors and interferon-gamma differentially affect cell surface molecules shared by monocytes and neutrophils. *Clin Exp Immunol* 1990, 81:339–345
33. Meagher LC, Savill JS, Baker A, Fuller RW, Haslett C: Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B<sub>2</sub>. *J Leukoc Biol* 1992, 52:269–273
34. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM: Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* 1998, 101:890–898